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STABILIZATION OF *Serratia marcescens* AGAINST FREEZE DRYING OR AEROSOLIZATION BY METAL-BINDING SOLUTES

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FOREWORD

I am happy to acknowledge the competent assistance of the following associates: Mr. Robert E. Lee, Jr.; Mr. Paul Rhoades, who generously made available and operated the freeze dryer; and Dr. John Ladino, who performed the urea analyses. The aerosol tests on the mixed slurries of Serratia marcescens and Bacillus subtilis were performed by Technical Evaluation Division. In particular, the stimulating discussions and criticisms of Mr. George Hess and Mr. Milton Shon were of valuable assistance.

ABSTRACT

Studies of the effects of solutes other than sugars upon the survival of Serratia marcescens subjected to freeze drying or aerosolization showed that the aerosol stability of that organism was markedly improved in the presence of some metal-binding compounds. In combination with minimally penetrable (MP) sugars, these solutes rendered the cells nearly as aerosol-stable as spores of Bacillus subtilis var. niger.

Freeze-drying stability of S. marcescens was enhanced (a) by some but not all of the aerosol-stabilizing metal binders, and (b) by various nitrogenous compounds, some of which supported the growth of the cells in chemically defined media.

Suitably chosen combinations of solutes proved capable of stabilizing S. marcescens both during aerosolization and during freeze drying.

From these data it was inferred that the stabilization of S. marcescens aerosolized at an unfavorable relative humidity depends upon the preservation of the integrity of metal-containing structures located in the cytoplasmic membrane. These structures may be protected (a) nonspecifically by an accelerated cellular dehydration induced by MP sugars, and (b) specifically, by metal-binding compounds that (reversibly) combine with the sensitive loci. In conbination, these two types of compounds exert synergistic protective effects.

The survival of freeze-dried cells, however, was ascribed simply to the maintenance of an optimum intracellular water content, created by the presence of freely penetrable (FP) solutes. Metal-binding was not a solute property that was necessary or sufficient for freeze-drying stabilization.

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TABLES

I. INTRODUCTION

The effects of various sugars on the survival of Serratia marcescens cells in suspensions subjected to freeze drying, or to aerosolization at unfavorable relative humidities, have been described recently.[1]* Freely penetrable** (FP) sugars stabilized the cells against freeze drying, presumably through their elevation of the water content of the stressed cells. Minimally penetrable** (MP) sugars protected aerosolized cells, an effect that was attributed to an acceleration of cellular dehydration. As expected, no single sugar, or mixture of sugars, protected the cells against both stresses.

It was subsequently observed that the stability induced by sugars was markedly modified in the presence of solutes other than sugars. This paper is a presentation of these observations.

II. MATERIALS AND METHODS

Some of the materials, methods, and equipment employed in these tests have been described previously, including (a) preparation of cell slurries, (b) freeze-drying techniques, and (c) aerosol test techniques.[1]

Chemically defined media were prepared for use in determining the ability of S. marcescens to grow on various nitrogenous compounds. The media contained the indicated concentrations of solutes: (a) 0.2 per cent Na citrate, 0.1 per cent KH_2PO_4, 0.05 per cent K_2SO_4, 0.025 per cent $MgSO_4 \cdot 7H_2O$, and 0.001 per cent $FeCl_3$; (b) 0.2 per cent glucose; and (c) 0.2 per cent of nitrogenous substrate. The solutions marked (a), (b), and (c) were combined after being autoclaved separately, then inoculated and incubated for 18 hours at 25°C.

As in the work reported previously, all aerosols were generated at 30 per cent relative humidity (RH) and 25°C.

One series of aerosol tests was performed on mixed slurries containing 20×10^9 S. marcescens cells and 2×10^9 Bacillus subtilis var. niger spores per milliliter. The B. subtilis organisms were grown in a casein acid digest medium, centrifuged to a heavy mud, and finally extruded into an acetone and dry ice bath. The resulting frozen strands were stored at -78°C until needed. Suspensions containing 20×10^9 spores per milliliter

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* See Literature Cited.

** Penetrable into the cell space bounded externally by the surface of the cell membrane.

were created by thoroughly blending 100 grams of frozen concentrate with 1600 milliliters of water, and then heating this material at 70°C for 15 minutes to kill any residual vegetative organisms. This suspension was diluted with nine parts of S. marcescens slurry and solutions of solute to yield mixed slurries of desired solute content and cell concentrations.

The numbers of viable S. marcescens and B. subtilis organisms in samples of the mixed slurries were determined from colony counts obtained by plating the suspensions, appropriately diluted, upon the following two media:

(a) Bacto-casitone agar plus 0.000025 per cent Brilliant Green; the dye suppressed the growth of B. subtilis but not that of S. marcescens colonies.

(b) Bacto-tryptose agar plus 0.005 per cent potassium tellurite; the tellurite suppressed the growth of S. marcescens but not that of B. subtilis colonies.

Chemicals used in this work were all of reagent grade or of the highest purity commercially available. All the compounds are commercially available from such sources as Fisher Scientific Co., Eastman Kodak Co., Nutritional Biochemicals Corp., and K and K Laboratories.

A. CRITERIA OF METAL-BINDING ACTIVITY OF SOLUTES

Solutes that combined with the ions of the metals of the first transition series (Fe, Co, Ni, Cu) or of the alkaline earths (Mg, Ca, Ba, Sr) were labeled "metal binders." Solutes were inferred to possess metal-binding activity upon the following different types of evidence:

(a) Many solutes were reported to be classically metal-chelating and metal-sequestering agents: dithizone, 8-hydroxyquinoline, the dithiocarbamates, and thiourea.

(b) Other solutes in the presence of metal ions yielded colored reaction products of unknown stability. Examples of this type included dipyridyl (in the presence of ferrous ions), Titan yellow (in the presence of magnesium), and dimethylglyoxime (in the presence of nickel).

(c) Still other solutes were capable of sequestering metals, primarily through the formation of highly insoluble and undissociated products. Sodium arsenite was such a compound; it apparently prevented the autoxidation of neutral solutions of ascorbic acid by removing the catalytically active copper and ferrous ions in this way.

B. SOLUTE PENETRABILITY DETERMINATIONS

The penetrability of solutes into S. marcescens was evaluated by adding known amounts of compounds to cell suspensions; this technique has been described in detail.1/ Concentrations of urea were determined by micro-Kjeldahl analysis. Concentrations of thiourea were determined colorimetrically by the use of Grote's reagent.2/

Concentrations of oxalyl dihydrazide (ODH) were determined colorimetrically as follows: 0.1 milliliter of 1 per cent $FeCl_3$ was added to five milliliters of solution containing ODH. The mixture was shaken for 15 seconds, 0.1 milliliter of 1N NaOH was added, and the resultant solution shaken vigorously for five minutes. The yellowish-brown color that developed upon addition of the NaOH was converted to green upon shaking; the intensity of color was proportional to the amount of ODH present (from 0 to 1000 micrograms per milliliter). Readings were made in a Coleman Junior Spectrophotometer at 540 millimicrons and at 630 millimicrons.

Concentrations of dithiocarbamates were determined from the intensity of color developed by their CCl_4-soluble copper salt.3/

III. RESULTS

A. EFFECTS OF PENETRABLE NON-SUGARS ON STABILITY OF AEROSOLIZED OR FREEZE-DRIED CELLS

Both urea and theourea were found experimentally to be freely penetrable (FP) into S. marcescens cells. The effects of these compounds on the survival of cells aerosolized or freeze dried from suspensions of a minimally penetrable (MP) sugar, raffinose, are shown in Table I. Urea and thiourea, like the FP sugars, increased the number of cells surviving after freeze drying; the combined effects of urea and thiourea were cumulative. The survival of cells aerosolized from suspensions containing raffinose was impaired in the presence of urea, another effect typical of a FP sugar. Thiourea, on the other hand, induced a remarkable enhancement of the stability of aerosolized cells, even in the simultaneous presence of urea.

TABLE I. EFFECTS OF ADDED THIOUREA AND UREA ON THE SURVIVAL OF VIABLE CELLS IN SUSPENSIONS SUBJECTED TO FREEZE DRYING OR AEROSOLIZATION

Solute Content of Cell Suspension		Survival, per cent		
1% Raffinose (0.017M) Plus		After Freeze Drying	After Aerosol Exposure for	
0.1% Urea (0.017M)	0.1% Thiourea (0.013M)		2 minutes	16 minutes
-	-	4	25	3
+	-	27	22	0.6
-	+	27	43	18
+	+	57	45	15

B. EFFECTS OF METAL-BINDING SOLUTES ON THE STABILITY OF AEROSOLIZED OR FREEZE-DRIED CELLS

S. marcescens was found to grow freely in chemically defined media containing urea as the sole source of nitrogen. Profuse growth was also obtained in media with urea plus thiourea, but thiourea alone did not support growth of the cells in these media.* These observations indicated thiourea was not a growth-inhibiting structural analogue of urea; therefore, it must have stabilized aerosolized cells through some companion property,

* Author's unpublished results.

such as its well-known metal-binding activity.[4-7] In order to explore this possibility, other suspensions containing raffinose plus some metal-binding compound were subjected to freeze drying and to aerosolization in the presence and absence of urea. The results of these tests are shown in Table II. Like thiourea, the other metal binders (a) stabilized aerosolized cells whether or not urea was present, and (b) except for sodium diethyldithiocarbamate, stabilized the cells against freeze drying. Metal binding was apparently the solute property correlated with aerosol-stabilizing activity.

TABLE II. MODIFICATION OF THE EFFECTS OF UREA ON THE SURVIVAL OF FREEZE-DRIED OR AEROSOLIZED S. MARCESCENS BY SOLUTES WITH METAL-BINDING PROPERTIES

Solute Content of Cell Suspension	Survival, per cent After Aerosolization				After Freeze Drying	
	As Is		Plus 0.1% Urea			
	After 2 Min.	After 16 Min.	After 2 Min.	After 16 Min.	As Is	With 0.1% Urea
1% raffinose	25	3	23	0.6	4	27
1% raffinose + 0.1% thiosemicarbazide	43	17	45	19	27	47
1% raffinose + <0.1% guanidine H_2CO_3	45	14	45	19	21	54
1% raffinose + 0.4% $NaAsO_2$ (pH to 7.0 w/HCl)	39	24	43	22	39	66
1% raffinose + 0.1% Na diethyldithiocarbamate	46	26	54	31	<1	2

Combinations of raffinose and many other compounds known to have metal-binding properties (or containing metal-binding structural configurations) were then tested as stabilizers for freeze-dried or aerosolized cells. Some of the compounds (Table III) remarkably improved the stability of cells aerosolized from suspensions containing raffinose. Most of these solutes were nitrogenous compounds, and many of them also contained sulfur. Two solutes, kojic acid and sodium arsenite, contained neither nitrogen nor sulfur. Another group of metal-binding solutes (Table IV), however, did not markedly enhance the survival of cells aerosolized in the presence of

TABLE III. EFFECTS OF COMPOUNDS WITH METAL-BINDING PROPERTIES ON THE SURVIVAL OF FREEZE-DRIED OR AEROSOLIZED S. MARCESCENS

Suspensions Contained One Per Cent Raffinose Plus	Survival, per cent		
	After Aerosol Exposure For 2 Minutes	After Aerosol Exposure For 16 Minutes	After Freeze Drying
No Additions, reference control	26	7	5
Sulfur Compounds			
0.1% thiourea	44	18	30
0.1% methylthiourea	49	33	31
0.1% thiosemicarbazide	50	33	27
0.1% 4-phenyl 3-thiosemicarbazide	47	27	8
<0.1% 2,3-butanedioneoxime thiosemicarbazone	53	26	10
0.1% Na dimethyldithiocarbamate	50	34	5
0.1% dimethylammonium dimethyldithiocarbamate	55	32	7
0.1% Na diethyldithiocarbamate	56	31	<1
0.1% diethylammonium diethyldithiocarbamate	52	26	1
Hydroxyquinolines			
0.1% 8-hydroxyquinoline	56	21	5
0.1% 5,7-dibromo-8-hydroxyquinoline	47	15	6
<0.1% 5-chloro-7-iodo-8-hydroxyquinoline	46	18	5
Hydrazides			
0.1% semioxamazide	50	25	5
0.1% oxalyl dihydrazide	55	30	7
0.1% carbohydrazide	53	23	7
0.1% semicarbazide	43	21	10
0.1% aminoguanidine H_2CO_3	47	23	18
Other Nitrogenous Compounds			
0.05% dipyridyl	49	23	16
0.05% o-phenanthroline	52	27	22
0.1% cupferron	51	32	12
0.1% biurea	53	26	6
0.1% 1,2,3-cyclohexanetrione trioxime	50	25	11
Compounds Without Nitrogen or Sulfur			
0.4% $NaAsO_2$ (H_2SO_4 to pH 7.0)	52	27	16
1.0% kojic acid (NaOH to pH 7.0)	49	25	69

TABLE IV. INABILITY OF SOME METAL-BINDING COMPOUNDS TO STABILIZE AEROSOLIZED S. MARCESCENS

Solute Content of Cell Suspension	Aerosol Survival, %	
	After 2 Min	After 16 Min
One Per Cent Raffinose	26	7
Plus		
<0.1% dithio-oxamide	39	8
0.5% EDTA, Na salt	29	2
<0.1% diphenylthiocarbazone	16	1
<0.1% dimethylglyoxime	20	4
0.1% ethylenethiourea	34	7
0.1% Na xanthogenate	45	6
<0.1% diphenylcarbazide	12	2
0.1% mercaptobenzthiazole	22	5
0.1% picolinic acid, Na salt	32	4
0.1% sulfosalicylic acid, Na salt	43	10
0.1% Titan yellow	42	3
0.1% 2,3-butanedione 2-oxime	25	4
0.1% furil dioxime	31	4
0.1% biquinoline	35	6
0.1% formylthiosemicarbazide	29	5
1.0% meconic acid, Na salt	37	7
1.0% dipicolinic acid, Na salt	18	3
0.1% iminotriacetic acid, Na salt	28	5

raffinose. These ineffective metal-binding solutes included such well-known and widely used reagents as EDTA, dithizone, and dithio-oxamide. Metal binding now appeared to be a solute property that was necessary but not sufficient for enhancement of aerosol stability.

Cellular survival after freeze drying was improved by some but not by all of the aerosol-stabilizing solutes shown in Table III; the ineffective compounds included the hydroxyquinolines, the dihydrazides, and the dithiocarbamates. Some simple nitrogenous compounds that enhanced freeze-drying recovery levels are shown in Table V. All these compounds were lacking in metal-binding or aerosol-stabilizing activity. In addition, they failed to support the growth of S. marcescens as the sole source of nitrogen in chemically defined media, either completely (acetylurea, hydrazine) or almost completely (formylurea, guanidine).* Thus, the solute property of metal binding appeared to be neither necessary nor sufficient to stabilize cells during freeze drying. Also, as previously observed among the sugars, metabolized and nonmetabolized nitrogenous compounds were equally effective as stabilizers for freeze-dried cells.

TABLE V. STABILIZATION OF FREEZE-DRIED CELLS BY NITROGENOUS SOLUTES LACKING METAL-BINDING OR AEROSOL-STABILIZING ACTIVITY

Suspensions Contained One Per Cent Raffinose Plus	Recovery of Viable Cells, per cent		
	After Aerosol Exposure for 2 min	After Aerosol Exposure for 16 min	After Freeze Drying
Nothing (Reference control)	26	7	5
0.1% Acetylurea	19	2	20
0.1% Formylurea	20	2	35
0.1% Guanidine HCl	5	1	20
0.1% Hydrazine hydrate	26	0.2	45

C. EFFECTIVENESS OF AEROSOL STABILIZATION BY SOLUTE COMBINATIONS

The extent to which aerosolized S. marcescens was stabilized by combinations of MP sugars and metal binders was determined in trials made on slurries containing S. marcescens and spores of B. subtilis in the presence

* Author's unpublished results.

of raffinose plus thiourea plus sodium ascorbate. The spores were reported to possess complete biological stability, disappearing from aerosols at the same rate as the radioisotope tracers.[8] In these tests, the aerosol recovery values for the vegetative S. marcescens cells and the B. subtilis spores (Table VI) were fairly close to one another, indicating that near-maximal levels of biological stability had been induced in S. marcescens.

TABLE VI. RELATIVE STABILITY OF S. MARCESCENS AND SPORES OF B. SUBTILIS IN MIXED AEROSOLS

Solute Content of Cell Suspension	Organism Evaluated	Aerosol Survival, per cent After 2 min	After 16 min	After 30 min
0.4% $NaAsO_2$ plus 1% ascorbic acid plus NaOH to pH 7.0	S. marcescens	50	32	23
	B. subtilis	41	32	26
1% raffinose plus 0.4% $NaAsO_2$ plus 1% ascorbic acid plus NaOH to pH 7.0	S. marcescens	40	27	18
	B. subtilis	38	30	25

D. EFFECTS OF METAL-ION REMOVAL BY NONSOLUTES

The free polyvalent metal-ion content of cell suspensions containing raffinose was depleted by two different methods: (a) centrifuging the suspensions, discarding the supernatant fluid, and resuspending the cells in fresh raffinose solution; or (b) adding a cation-exchange resin (Dowex 50 in the sodium form) to the suspension. These treatments did not improve the aerosol stability of the cells (Table VII). Thus, the sequestering of extracellular transition metal ions in solutions did not appear to be the metal-binding process that induced aerosol stability.

TABLE VII. EFFECTS OF WASHING OR ION-EXCHANGE-RESIN TREATMENT ON THE AEROSOL STABILITY OF S. MARCESCENS

Cell Suspension		Aerosol Survival, per cent	
Solute Content	Treatment	After 2 min	After 16 min
1% raffinose	None	40	10
1% raffinose with 1% NaOH plus Dowex 50 resin (H form) to make pH 7.0	None	31	8
1% raffinose with 5% NaOH plus Dowex 50 resin (H form) to make pH 7.0	None	36	6
1% raffinose	Cells sedimented and resuspended in fresh 1% raffinose solution	34	10

E. EFFECTS OF WATER DISTRIBUTION

The effectiveness with which metal binders stabilized aerosolized cells was controlled by the kinds and amounts of other solutes in the cell suspensions. The metal binders were maximally effective only in the presence of "favorable water distributions" created by (a) the presence of MP sugars and (b) the absence of FP sugars or other FP solutes. The data that led to these inferences are as follows:

(a) Suspensions containing varied amounts of thiourea (FP) and one per cent raffinose (MP) were subjected to freeze drying or aerosolization (Table VIII). Freeze-drying survival was higher with one per cent than with 0.1 per cent thiourea, indicating that one per cent thiourea could not very well be toxic. One per cent thiourea, however, yielded relatively poor aerosol recovery compared with 0.5 or 0.1 per cent. This effect was attributed to the extreme retardation of cellular dehydration induced by the presence of too much of the FP solute, thiourea. It may be pointed out that most of the cells in the one per cent thiourea preparation died prior to the two-minute sampling, an effect consistent with a death-during-drying hypothesis: Those cells that survived the first two minutes of aerosol life showed excellent aerosol stability.

TABLE VIII. IMPAIRMENT OF AEROSOL STABILITY OF S. MARCESCENS BY EXCESSIVE CONCENTRATIONS OF THIOUREA

Solute Content of Cell Suspension	Recovery of Viable Cells, per cent		
	After Aerosol Exposure		After Freeze Drying
	For 2 min	For 16 min	
1% raffinose	41	17	5
Plus			
0.1% thiourea	52	36	27
0.5% thiourea	52	35	67
1.0% thiourea	19	11	51

(b) The survival of S. marcescens after aerosolization in the presence of raffinose (MP), sorbose (FP), and the metal binder sodium arsenite is shown in Table IX. The highest levels of aerosol survival were obtained in the presence of arsenite plus the MP sugar, raffinose. The effectiveness of arsenite as an aerosol stabilizer was largely dissipated, however, when (a) raffinose was omitted, or (b) sorbose was added to the cell suspensions. These data also indicated that maximal aerosol stability was induced by metal binders only in the presence of MP sugars and the absence of FP solutes.

(c) The survival of S. marcescens after aerosolization in the presence of metal-binding aerosol stabilizers with no other added solute is shown in Table X. All of these solutes (but one) were employed at or below 0.1 per cent concentration, and many of them were soluble in water only to a limited extent. It seems improbable, therefore, that these compounds could stabilize aerosolized cells as the MP sugars did, by accelerating their dehydration. Nevertheless, all but 2 of the 22 metal binders listed in Table X improved the stability of aerosolized cells; the improvement varied from slight to unexpectedly great. The aerosol stability induced by these solutes in the presence of raffinose was presented earlier (Table III). Comparison of the values in Tables III and X reveals how greatly the effectiveness of the metal binders as aerosol stabilizers is enhanced in the presence of raffinose.

TABLE IX. MODIFICATION OF THE AEROSOL-STABILIZING EFFECTS OF METAL-BINDING SOLUTES BY SUGARS OF DIFFERING PENETRABILITY

Solute Content of Cell Suspensions	Aerosol Survival, per cent	
	After 2 min	After 16 min
Water	7	0.2
Water plus one per cent sorbose[a]	7	0.06
One per cent raffinose[b]	40	9
One per cent raffinose plus one per cent sorbose	18	0.2
0.4 per cent sodium arsenite[c]	13	3
0.4 per cent sodium arsenite[c] plus one per cent sorbose	21	1
One per cent raffinose plus 0.4 per cent sodium arsenite[c]	44	18
One per cent raffinose plus 0.4 per cent sodium arsenite[c] plus one per cent sorbose	29	1

a. Sorbose was shown previously to be freely penetrable (FP) into cells.
b. Raffinose was shown previously to be minimally penetrable (MP) into cells.
c. pH adjusted to 7.0 with HCl.

TABLE X. SURVIVAL OF S. MARCESCENS AFTER AEROSOLIZATION IN THE PRESENCE OF METAL-BINDING SOLUTES

Added Solutes in Cell Suspension	Survival, per cent	
	After 2 min	After 16 min
None	9	0.5
0.1% thiourea	7	3
0.1% methylthiourea	7	2
0.1% thiosemicarbazide	19	9
0.1% 4-phenyl 3-thiosemicarbazide	8	0.5
0.1% 2,3 butanedioneoxime thiosemicarbazone	26	6
0.1% sodium diethyldithiocarbamate	9	5
0.1% 8-hydroxyquinoline	22	3
0.1% 5,7-dibromo-8-hydroxyquinoline	11	0.3
0.1% 5,7-chloriodo-8-hydroxyquinoline	17	2
0.1% semioxamazide	21	6
0.1% oxalyl dihydrazide	19	7
0.1% carbohydrazide	27	16
0.1% aminoguanidine H_2CO_3	15	8
0.05% dipyridyl	22	5
0.05% o-phenanthroline	14	2
0.1% cupferron	12	3
0.1% biurea	13	1
0.1% 1,2,3 cyclohexanetrione trioxime	16	5
0.1% sodium arsenite (plus H_2SO_4 to pH 7.0)	11	4
1% kojic acid (plus NaOH to pH 7.0)	25	4
0.1% semicarbazide (free base)	18	9

F. PENETRABILITY OF METAL-BINDING SOLUTES

The penetrability of most of the metal binders listed in Table III was not determined. One reason was that standard methods were not available for the analytical determination of many of these solutes. Also, compounds such as the dithiocarbamates yielded penetrability values far in excess of 100 per cent, probably because they were adsorbed and concentrated by the cells. The penetrability of only one solute was established with assurance, namely oxalyl dihydrazide (ODH). This compound was MP, because only 24 per cent of the cell volume* was available for its dilution.

* Volume within the cell wall.

IV. DISCUSSION

A. STABILIZATION OF FREEZE-DRIED CELLS

The stabilization of freeze-dried cells by FP sugars was attributed to a solute-induced enhancement of their intracellular water content (IWC). This hypothesis was extended to account for the stabilizing effects of non-sugar solutes by postulating that

(a) Cells survived freeze drying only when their IWC was maintained, and

(b) IWC was increased progressively in the presence of increasing amounts of all FP solutes and competitively diminished in the presence of MP solutes.

The concept that all FP solutes act to increase the IWC of freeze-dried cells requires some discussion. The effectiveness of FP sugars in stabilizing freeze-dried cells was ascribed to their "water-binding" or syrup-forming properties. Solutes in general, however, are soluble because their molecules unite chemically with water molecules to form hydrates. In this sense, all solutes may act to bind water molecules; this mechanism is believed to account for the effectiveness of FP nitrogenous solutes (such as urea and thiourea) as freeze-drying stabilizers.

This hypothesis has ascribed the effects of freeze-drying stabilizers to their FP properties, although penetrability determinations were made on very few of these solutes. In defense of this hypothesis, one may indicate that all of the non-metal-binding freeze-drying stabilizers, like the FP sugars, impaired the stability of aerosolized cells. Furthermore, the freeze-drying stabilizers were a heterogeneous group, which could not obviously be classified by any other common property: They included inorganic ($NaAsO_2$) and organic compounds, sugars and nitrogenous compounds, metal binders and non-metal binders, and nutrient and non-nutrient molecules.

B. STABILIZATION OF AEROSOLIZED CELLS

Metal-binding solutes appeared to stabilize aerosolized cells directly and not through their effects on metal ions in the suspending fluid. These solutes were maximally effective as stabilizers in the presence of MP sugars, whose stabilizing activity had previously been ascribed to their acceleration of cellular dehydration. It is now proposed that vital metal-containing structures in the cytoplasmic membrane are the parts of the cell that are most sensitive to aerosol damage. These structures may be stabilized against aerosol exposure at unfavorably low humidities: (a) nonspecifically, by MP sugars, which accelerate the rate and/or extent of cellular dehydration;

and (b) specifically, by metal-binding agents, which combine reversibly with their metal atoms. The sensitive metal-containing loci are tentatively located in the cytoplasmic membrane because many of the aerosol-stabilizing metal binders did not also stabilize freeze-dried cells. On the basis of previous assumptions, such solutes could not have been FP; therefore their activity must have been limited to the cell surface, which can reasonably be considered to be the cytoplasmic membrane. (One metal-binding agent, oxalyl dihydrazide, was shown to be MP, an observation that supported the above reasoning.)

The identity of the metal with which the metal binders supposedly combine is not certain. However, the growth of S. marcescens was restricted in media rendered deficient in iron.[9] Organic tightly bound bound iron occurs in many essential cell components; for example, in metalloflavoproteins such as xanthine oxidase, and in porphyrin-containing enzymes such as the cytochromes, peroxidase, and catalase. Inspection of the list of aerosol-stabilizing metal binders (Table III) revealed the presence of many reagents well-known to react with iron, including dipyridyl, phenanthroline, 8-hydroxyquinoline, the dithiocarbamates, thiourea, cupferron, and thiosemicarbazide. Also, the results of another investigation[10] suggest that copper salts killed S. marcescens, in the presence of ascorbic acid, through a chemical reaction with sensitive iron atoms in or on the cells.

Therefore, since iron is one of the few metals needed by the cell for growth, and since many of the metal-binding aerosol stabilizers have an affinity for iron, it is suggested that the metal binders may be combining with vital cellular iron atoms and so lessening their sensitivity toward denaturation or destruction during aerosolization.

C. STABILIZATION OF CELLS AGAINST BOTH FREEZE DRYING AND AEROSOLIZATION

It has been assumed that solute-induced changes in IWC could only improve freeze-drying stability at the expense of aerosol stability and vice versa. Suspensions containing raffinose plus thiourea, however, were stabilized during both freeze drying and aerosolization. The mechanisms that produced this desirable effect are believed to be these: FP thiourea, like urea, stabilized freeze-dried cells by elevating their IWC. During aerosolization, however, the potentially adverse effects of the FP solute were completely overcome by (a) the plasmolyzing effects of MP raffinose plus (b) the protective activity of the metal binder, thiourea, upon sensitive cellular loci.

Several metal binders shared with thiourea the ability to stabilize cells during both freeze drying and aerosolization. Additional experimental evidence is required to support the assumption that these metal binders are all FP solutes.

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